ULTRASTRUCTURAL DAMAGE TO ARTIFICIALLY FROZEN CITRUS LEAF TISSUE

By

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	x
INTRODUCTION	1
LITERATURE REVIEW	3
Mechanism of Injury	3
Thermal Shock	3
Solute and Dehydration Injury	4
Mechanical Injury	6
Thawing and Rehydration Injury	7
Disulfide Hypothesis	8
Cellular Membranes	9
Structure	9
Membrane Injury	10
Freezing Events	13
Cryoprotection	15
Chemical	15
Natural	18
Freezing in Citrus	20
MATERIALS AND METHODS	25
Plant Materials	25
Leaf Freezing Point Determination	26
Tissue Selection and Transportation	27
Tissue Preparation for Ultra-Thin Sectioning	27
Tissue Preparation for Freeze-Etching	28
Freeze-Etching	29
Treatment Comparisons	30

TABLE OF CONTENTS (cont'd)

	Page
RESULTS AND DISCUSSION	31
Mean Leaf Freezing Points	31
Ultra-Thin Sections	33
Freeze-Etching of Citrus Leaf Tissues	51
Freeze-Etch Replication of Solutions	51
Initial Ice Formation in Leaf Tissues at 2°C Above MLFP	59
Intracellular Ice Formation in Leaf Tissues at 2°C Below MLFP	78
Localized Cellular Disruption at 2°C Below MLFP	87
Membrane Disruption of Hardy Varieties	102
SUMMARY AND CONCLUSIONS	112
LITERATURE CITED	115
BIOGRAPHICAL SKETCH	134

LIST OF TABLES

Table		Page
1	Mean leaf freezing points (MLFP) of citrus plants acclimated in a growth chamber. . . .	32
2	Summary of the final cooling temp maintained on the thermoelectric plate for each citrus cultivar prepared for freeze-etching (°C)	69

LIST OF FIGURES

Figure		Page
1	Electron micrograph of unhardened palisade cells of 'Tahiti' lime	35
2	Electron micrograph of unhardened palisade cells of 'Marsh' grapefruit	37
3	Electron micrograph of unhardened palisade cells of 'Orlando' tangelo	39
4	Electron micrograph of unhardened palisade cells of 'Owari' satsuma	41
5	Electron micrograph of hardened palisade cells of 'Tahiti' lime	43
6	Electron micrograph of hardened palisade cells of 'Marsh' grapefruit.	45
7	Electron micrograph of hardened palisade cells of 'Orlando' tangelo	47
8	Electron micrograph of hardened palisade cells of 'Owari' satsuma	49
9	Freeze-etched replica of 5% glutaraldehyde solution	53
10	Freeze-etched replica of double-distilled water supercooled to -10.6°C and maintained at that temp for 15 min.	55
11	Freeze-etched replica of double-distilled water frozen from room temp.	58
12	Freeze-etched replica of glycerol pretreated 'Tahiti' lime leaf tissue cell	62

LIST OF FIGURES (continued)

Figure		Page
13	Freeze-etched replica of glycerol pretreated 'Marsh' grapefruit leaf tissue cell.	64
14	Freeze-etched replica of glycerol pretreated 'Orlando' tangelo leaf tissue cell . . .	66
15	Freeze-etched replica of glycerol pretreated 'Owari' satsuma leaf tissue cell	68
16	Freeze-etched replica of glutaraldehyde pretreated 'Tahiti' lime leaf tissue frozen at MLFP +2°C.	71
17	Freeze-etched replica of glutaraldehyde pretreated 'Marsh' grapefruit leaf tissue frozen at MLFP +2°C	73
18	Freeze-etched replica of glutaraldehyde pretreated 'Orlando' tangelo leaf tissue frozen at MLFP +2°C	75
19	Freeze-etched replica of glutaraldehyde pretreated 'Owari' satsuma leaf tissue frozen at MLFP +2°C	77
20	Freeze-etched replica of glutaraldehyde pretreated 'Tahiti' lime leaf tissue frozen at MLFP -2°C.	80
21	Freeze-etched replica of glutaraldehyde pretreated 'Marsh' grapefruit leaf tissue frozen at MLFP -2°C	82
22	Freeze-etched replica of glutaraldehyde pretreated 'Orlando' tangelo leaf tissue frozen at MLFP -2°C	84
23	Freeze-etched replica of glutaraldehyde pretreated 'Owari' satsuma leaf tissue at MLFP -2°C	86

LIST OF FIGURES (continued)

Figure		Page
24	Freeze-etched replica of 'Tahiti' lime with cell wall intact with frozen surroundings at 2°C below MLFP	89
25	Freeze-etched replica of 'Tahiti' lime exhibiting ice crystals in the cell wall at 2°C below MLFP	91
26	Freeze-etched replica of 'Orlando' tangelo demonstrating intact chloroplast with surrounding frozen cytoplasm at 2°C below MLFP . .	95
27	Freeze-etched replica of 'Owari' satsuma demonstrating intact chloroplast with surrounding frozen cytoplasm at 2°C below MLFP .	97
28	Freeze-etched replica of 'Orlando' tangelo demonstrating ice crystal damage in a chloroplast frozen at 2°C below MLFP	99
29	Freeze-etched replica of 'Orlando' tangelo frozen at MLFP -2°C in water exhibiting plasma membrane damage.	104
30	Freeze-etched replica of 'Owari' satsuma frozen at MLFP -2°C in water exhibiting plasma membrane damage	106
31	Freeze-etched replica of 'Orlando' tangelo glycerol control showing intact plasma membrane.	108
32	Freeze-etched replica of 'Owari' satsuma glycerol control showing intact plasma membrane.	110

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

ULTRASTRUCTURAL DAMAGE TO ARTIFICIALLY FROZEN CITRUS LEAF TISSUE

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No ultrastructural changes were observed in comparing cold-hardened and unhardened leaf cells of 'Tahiti' lime, 'Marsh' grapefruit, 'Orlando' tangelo and 'Owari' satsuma using ultra-thin sections viewed with an electron microscope

Initial ice crystal formation in all 4 cultivars was intercellular. Leaf tissues of the cold-hardened and unhardened varieties were frozen to 2°C above their mean leaf freezing point (MLFP) on a thermoelectric cooler, freeze-etched, then viewed under a transmission electron microscope.

'Tahiti' lime and 'Marsh' grapefruit were supercooling whereas 'Orlando' tangelo and 'Owari' satsuma were resistant to intracellular ice nucleation Tissues cooled to 2°C below the MLFP demonstrated a broad range of injury from

intracellular ice formation which corresponded with reported cold tolerance of each cultivar.

All 4 cultivars displayed some degree of ice crystal formation within the cell wall, but its formation was not damaging at the temp studied. The crystals were small and randomly scattered throughout the wall structure.

Large ice crystal growth through adjoining tissues, membranes and organelles was not observed. There appeared to be a specific temp at which nucleation and subsequent small ice crystal formation began and this temp was different in the various cellular constituents.

Chloroplasts of the hardy cultivars of 'Orlando' tangelo and 'Owari' satsuma displayed little damage as a result of internal ice crystal formation. Cells cooled to -10.5°C show ice formation surrounding the chloroplast, but the chloroplast contained no ice crystals.

Plasmolysis disruption was observed in all cultivars at 2°C below the MLFP. Tender cultivars displayed total disruption, whereas the hardy cultivars displayed sporadic disruption.

INTRODUCTION

The actual mechanism of freeze damage in citrus is unknown. Citrus tissues, if cooled slowly, will supercool, the degree depending on the variety. When ice crystal nucleation begins, formation is thought to occur extracellularly. It is this extracellular ice formation that gives citrus leaves their so-called 'water soaked' appearance. Upon rewarming, the water soaked area will disappear, with partial or complete recovery of leaf tissues. This recovery leads one to believe that initial formation of extracellular ice is not responsible for freeze damage. Damage is thought to be due to the formation of intracellular ice. As of yet, no one has directly demonstrated the presence of ice crystals within the protoplasm, but some researchers, using the water-alcohol substitution method, have located areas of cytoplasmic and membrane disruption in frozen cells (180, 186). These areas are thought to be a result of crystalline ice.

The objectives of this research were to determine the location of initial intracellular and extracellular ice crystal formation in leaves of 4 different citrus cultivars,

and to observe the influence of plant-hardening temp regimes on the size, shape and location of these ice crystals. Concomitantly, observations were made on specific cellular membranes and organelles to detect any changes which might result from exposure of the plants to different plant-hardening temp regimes.

LITERATURE REVIEW

Mechanism of Injury

Generally, supercooling cells for short periods of time will produce no cellular injury, although specific cells are very susceptible to low temp (2). Using thermodynamical osmolal conc formulae, it has been determined that the cytoplasm should freeze at $-1^{\circ}C$, but it also has been shown that the temp of the cytoplasm can be lowered to -11° to $-16^{\circ}C$ without freezing even when ice is present in the external media, indicating that the cellular membranes somehow prevent ice formation within the cell (87).

There are 2 separate events which cause freezing injury: (1) physical-chemical events associated with the phase change of liquid water to ice and (2) mechanical injury caused by ice crystal growth (84, 90, 163)

Thermal Shock

Slow cooling without ice formation is not damaging to most cells, but rapid chilling to temp above or below $0^{\circ}C$ can be decidedly injurious. Such injury is referred to as

thermal shock (86). The causal agent is unknown. Possibly, damage to the lipoprotein complex in cell membranes is involved, which disrupts cellular permeability (57, 147). Thermal shock is not a universal occurrence and appears to require specific conditions (137). Escherichia coli is highly susceptible to this phenomenon in early stages of logarithmic growth but is almost completely resistant in late logarithmic and all stationary growth phases (96). If thermal shock is a major factor in freeze injury to cells, damage should occur regardless of whether or not the suspension medium contains ice. This has been found not to be the case in the few instances in which direct comparisons have been made. Thermal shock is therefore not a major factor in freezing injury (86, 89, 90).

Solute and Dehydration Injury

A major problem in understanding solute injury is that 4 distinct events happen simultaneously: (1) liquid water is removed as ice, (2) solutes of low and high molecular weights concentrate, (3) cell volume decreases and (4) solutes precipitate. Each of these events, at one time or another, was thought to be the main cause of freeze damage. Unfortunately, all except solute precipitation are monotonic

functions of temp, thus they occur simultaneously during freezing (1, 2, 55, 56, 57, 65, 66, 68, 86, 90, 94, 95).

As protoplasmic temp drop below 0°C the cell water suspension begins to freeze. During this time pure ice separates out of solution and concomitantly the solutes in the residual liquid solution become increasingly conc. Ideal solutions of nonelectrolytes will conc by one molal unit for each 2°C drop in temp below its freezing point (86). In partially frozen solutions, the osmotic solute conc inside and outside the cell will equilibrate, if given sufficient time. These high solute conc then become lethal because electrolytes may modify the secondary and tertiary structure of macromolecules (66, 86, 89, 94). High solute conc may change cellular membranes by altering lipid structure, resulting in a change in permeability. They also may cause large changes in pH as various species of solute precipitate out below their eutectic point. Increases in the velocity of some chemical reactions may take place (66, 86, 163, 167). There is evidence that protein denaturation is not correlated with the number of degrees to solution is cooled below the eutectic point of the electrolytes, indicating that the amount of water removed from the protein

structure and not the solute concentration to denaturation (1, 2, 88).

Meryman (95) has recently suggested that cellular damage results from the inability of cells to dehydrate below a certain % of their normal volume. This shrinkage forms a transient pressure gradient across cellular membranes, which ultimately disrupts them causing leakage of cellular materials.

Mechanical Injury

The semiisolated compartmental nature of cells and tissues have made ice crystal formation and location studies extremely difficult. The methods utilized for these studies usually result in a disruption from the natural state. Since protoplasm may supercool to -10°C even when the extracellular medium is frozen, a higher vapor pressure is established inside the cell than outside, a situation which is thermodynamically unstable. Equilibrium will be accomplished in 2 ways: (1) supercooled water can flow out of the cell and freeze intercellularly or (2) the supercooled water can freeze intracellularly (86, 89, 94).

Ultimately mechanical injury caused by intracellular ice is a direct result of ice crystals exerting sufficient

force to disrupt the plasma membrane or membranes of various organelles (98). Intracellular freezing causes cell membranes to become leaky (82, 93, 97, 142, 143). A complicating note is that some cells subjected to intercellular ice have their membrane functions inactivated, opening the possibility that some temp labile factor is involved (26, 63, 140)

Thawing and Rehydration Injury

Freezing injury in higher plants may not occur during dehydration but during rehydration when cells are thawed (57, 93). Slow warming following freezing is often less harmful than rapid warming in plants (57, 156). Leaves from northern evergreen plants survived cooling to -45°C when warmed at 5°C/hr but failed to survive when warmed at 10°C/hr (110) Using birch and cherry twigs, damage was not found on rewarming from -60° to -5°C, but when there was rapid appearance of water in the cell between -5° and 0°C, cellular damage occurred (156) Bacteriophage T4R survived better if thawed slowly rather than rapidly. Basis for the damage was shown to be osmotic shock (52) A different situation is posed by slowly frozen yeast. Practically all deleterious effects of slow warming are below -60°C Long exposure time to conc solutes is cited as the damaging causal agent (91).

Disulfide Hypothesis

The disulfide hypothesis (56) is the only complete molecular model for freeze damage. It ascribes damage to events taking place during dehydration and rehydration (89). As water is converted to ice during freezing, structural proteins are forced into close proximity by the loss of water. As a consequence of the compaction, sulf-hydryl groups from within or on adjoining proteins become linked by disulfide bonds. As the cell is rehydrated competition between hydrogen bonds and disulfide bonds causes the protein to become denatured (56, 57, 168). The theory is said to account for nearly all observations of freezing injury, and for biochemical alterations in freeze-killed cells. The biochemical changes accompanying frost-hardening are those which would tend to reduce chances of disulfide bond formation. Cellular cryoprotectants would act in a similar manner (56, 57).

There are a number of difficulties with this theory (a) The number of disulfide bonds increases with freezing injury, but there is no evidence that these bonds form prior to injury, which must happen if this theory is correct. (b) It cannot explain the fact that rapid thawing increases

the number of survivors in rapidly cooled animal cells. (c) It does not account for the protective action of certain large molecular weight compounds which are unable to penetrate the cell to prevent shrinkage during freezing (89).

Cellular Membranes

Structure

Two models of membrane structure are presently in favor: (a) molecular layering (sandwich) and (b) molecular aggregates (particulate composition). It is generally agreed that membranes are not solid but rather fluid in structure. They are more or less composed of about equal parts of protein and lipids, interspersed with asymmetrically distributed carbohydrates and other prothetic groups (6, 7, 13, 25, 40, 46, 102, 164, 165). Extensive studies have revealed that the lipids contain a heterogeneous group of fatty acids. Generally the bulk of lipids are glycerophosphatides combined with various proportions of glycolipids, sterols, and neutral glycerides (25, 37, 39, 40, 81, 83). The protein fraction is of an extremely heterogeneous nature which has led to a descriptive classification of these proteins: (a) structural, (b) catalytic (enzymes), (c) extrinsic

(surface), easily extracted, (d) intrinsic (internal), not easily extracted, (e) transmembrane (penetrates entire membrane structure) and (f) amphipatic, which displays both hydrophobic and hydrophyllic properties depending on association with lipid fraction (6, 7, 102, 141). The precise arrangement between the protein and lipids is unknown; however, many plausible schemes have been developed (6, 7, 16, 116, 141, 152). Membrane structure is specific for a particular organelle association and function, thus membranes are not uniform in composition within a cell. Membranes associated with chloroplasts and mitochondria, as an example, have a distinct outer and inner structure which are separate in function and composition. The endomembranes (nuclear envelope, vacuole, endoplasmic reticulum) appear to be able to withstand physical stresses and contain specific sites for specialized enzymatic activity (3, 5, 18, 23, 37, 39, 40, 79).

Membrane Injury

Membrane damage may be the prime agent in causing death after a cell has been exposed to low temp (24, 26, 28, 68, 143). Within a given cell, not all membranes are damaged at the same temp or in the same manner (47, 71).

Cellular membranes tend to prevent intracellular ice nucleation at temp between -15° and 0°C (87, 90). Assuming a water-filled pore structure, ice crystals small enough to penetrate the water matrix of the membrane cannot exist above -10°C due to the high water activity produced by the small curvature radius of these crystals (71, 87, 130).

When ice crystal formation takes place in cells, the membranes are affected in 2 ways. (1) changes in permeability and (2) mechanical damage. The exact nature of permeability disruption is unknown, but the resulting leakage of cellular electrolytes is well established (7, 76, 80, 88, 90). Cellular leakage up to 20 times greater than normal has been observed in chilled plants (38, 62, 108). Permeability changes seem to result from a phase change in membrane lipid structure (112, 113, 148). Evidence indicates that the lipid content of membranes controls the temp of this phase shift (120). In mitochondria this phase shift from a fluid matrix to a solid gel takes place at 10° to 12°C (50, 77, 78, 118, 121). During this phase shift the mitochondrial membrane loses the ability to discriminate against protons (26). Changes in a membrane's physical state have been shown to influence membrane bound enzymatic

activity (27, 77, 82, 119, 138, 153) None of these phase transition shifts have been demonstrated in chilling-resistant species of plants (76).

The fatty acid composition of the membrane lipids of membranes appears to be correlated to chilling sensitivity in plants. It is unclear whether the fatty acids in membranes determine the physical phase transition or if other membrane components influence this transition (27, 64, 76, 162, 171). Plants originating from warm climates contain greater amounts of fatty acids in their lipids (76, 108).

Predisposition to membrane flexibility or ability to swell between hardy and nonhardy plants has been observed. Mitochondrial membranes of frost-hardy plants demonstrate a greater flexibility, thus swell to a greater extent than nonhardy plants when subjected to various osmotic solutes (78).

Mechanical membrane damage caused by ice crystal formation has been noted in many plants. This type of injury causes changes in membrane permeability (76, 80, 99, 143) with the result that large molecular weight molecules are able to penetrate (143). Frozen and thawed plasmolyzed of apply cork parenchyma cells allowed the protoplasm to

flow through the membrane and become deposited on the interior side of the cell wall (47).

Freezing Events

The cooling rate of cells or tissues depends on the ability of surrounding media to remove heat. Ice does not spontaneously form as the temp drops to the freezing point of water. Initially the cell and surrounding medium supercool, resulting in extracellular ice nucleation (54, 73, 107). The cell membrane excludes ice from the cell interior, and, until about $-10^{\circ}C$ is reached, the cell remains supercooled and unfrozen (2, 57, 87, 88, 98).

Liquid water has a greater chemical potential than ice, and as a consequence water will move from the interior to the exterior of the cell (85). The preceding dehydration produces an increase in cellular solute conc which, in turn, decreases the internal aqueous vapor pressure. This differential aqueous vapor pressure between the interior and exterior of the cell causes liquid water to flow out of the cell (80, 89, 107).

High cellular solute conc may ultimately produce a disruption of membranes and various chemical bonds. Dehydration causes an increase of internal cellular osmotic

pressure by approx 12 atmospheres effective negative pressure for each degree drop below 0°C (19). As the cell subcools, resulting internal stresses may become great enough to rupture chemical bonds causing denaturation of specific protains and membrane changes (80). As the cell dehydrates some solutes will precipitate out of solution. Precipitation may also occur if the temp is lowered below the solute's eutectic point (84, 134, 170). This precipitation of solutes will cause changes in protoplasmic pH.

Intracellular nucleation eventually takes place with the formation of many small ice crystals. These small crystals have higher surface free energy than large spherical crystals, resulting in the formation of large crystals from small ones. The formation of large crystals may result in mechanical injury of the cell (2, 80, 89, 90, 94).

Upon rewarming, the events will be approx reversed, resulting in water flow into the cell. The rate of water flow will depend on the permeability of the cell to water and the rewarming rate (72, 88, 94, 123).

Tender plants demonstrate chilling injury at temp of about -4° to -5°C. Primarily, damage is due to cell membrane disfunction. Plants such as lettuce and peas may

survive these temp even if ice is present in the plant tissues. Corn and beans will survive only if cellular water supercools without extracellular ice formation (80). Cell survivability is influenced by the dewpoint of the atmosphere and % water content of the plant (97, 129, 130, 131, 132).

Frost-hardy plants tolerate ice crystal formation and growth in intercellular cavities at tempt below -5°C. Injury to this type plant is probably due to dehydration resulting in chemical bond changes (133).

All plants are susceptible to enzyme inactivation when exposed to low temp. Specifically, the electron transport system in photosynthesis is easily disrupted by freezing. Many investigations are currently underway to determine the cause and extent of the damage (25, 28, 105, 148, 167, 177).

Cryoprotection

Chemical

Various unprotected cells can withstand freezing if suspended in pure water or simple single salt solutions and cooled at the cell's optimum cooling rate (90). Unfortunately most natural cooling does not take place at these optimum rates. The combination of low minimum temp and

undesirable cooling rates cause cellular death, presumably by exposure to conc solutes (88).

It was discovered using animal tissues that suspending cells in low conc of glycerol and dimethyl sulfoxide solutions substantially increased cell tolerance to low temp (69, 118). It was hypothesized that these chemicals protected, by reducing the cellular electrolyte conc and acting as a salt buffer (17, 65, 66, 122). The assumption was made that all cell solutes are electrolytes. From this assumption calculations were made using osmolar ratios of protectant to electrolyte, and it was theorized that only low molecular weight hydrophilic solutes forming solutions with a low eutectic point would protect cells (66, 95, 103). Low molecular weight cryoprotectants were generally thought to act on the basis of their conc and their ability to penetrate the cell (65, 104). Problems in this explanation arose with the discovery of large molecular weight cryoprotectants. Macromolecules such as polyvinyl pyrrolidone, polyethylene glycol, polyhydric alcohols and sugar solutions have demonstrated the ability to protect both animal and plant cells against freeze damage (66, 75, 106, 128). These observations present a strong case against protection by colligative effects

with the protoplasm, but they do not exclude this effect as a factor.

A possible alternate mode of action is that these macromolecules exhibit a preferential binding to water. This binding would reduce the amount of free water in the cell and raise the conc of electrolytes found in the free water fraction (60, 90).

Recent speculation has attributed cryoprotective action to the protectant's ability to discriminately form hydrogen bonds or to stabilize clathrate water structure. As of yet both are still speculation (15, 103, 104). Chemical cryoprotectants have the ability to raise the glass transformation point (Tg) of water by as much as 50°C. Thus when cooling or rewarming cells there is a much smaller temp range in which ice crystals are able to form (74, 75).

Recently the proposed method of protection for glycerol and dimethyl sulfoxide has been questioned. It was shown that the cryoprotection by using glycerol in fairly high conc was the result of cellular dehydration. If glycerol entered the cells at these conc, damage to the cell would result (137). Penetration into the cell is not necessary for protection of animal tissues by glycerol and

dimethyl sulfoxide (53). Thus increased cell survival by use of chemical cryoprotectants may chiefly depend on the protection of the cell surface (90).

Natural

Certain plants, when exposed to specific environmental conditions undergo natural changes which result in the initiation of a cryoprotective mechanism. These changes are directly related to the hardiness of the plant and involve changes in biochemical and physiological processes.

Plant hardiness is initiated by factors that reduce plant growth, especially short photoperiods and low temp (31, 32, 45, 61, 109, 125, 126, 127, 145, 155, 157). Hardiness may be modified by specific phytohormones. Gibberellic acid inhibits the induction or reduces the degree of hardiness (32, 154). Growth retardants such as Amo 1618, and the B-complex induce hardiness in plants even those grown under nonhardening environmental conditions (32, 158, 159).

Correlations between levels of specific plant cell substances and degree of hardiness have been observed There exists an interrelationship between plant hardiness and conc of reducing sugars within plant tissues (57, 61,

66, 106, 111, 125, 126, 128, 169, 184). Increased freezing resistance has been obtained by immersion of plant tissues into solutions of specific sugars (58, 124). Notable increases in cellular soluble proteins seem to coincide with the increase of hardiness in plants (36, 61, 135, 169). Often accompanying this rise in soluble protein is a rise in total RNA content (36). Many other correlations are observed when plant hardiness increases. The most interesting of these are the general quantitative increases in lipid content, organic acids, DNA, anthocyanins, and nicotinamide adenine dinucleotide (36, 51, 41, 61, 111).

Multiple correlations existing during hardening have been investigated. The better established of these are between sugars and sulfhydryl content; soluble protein and pH; organic acids and amino acids, phosphorus, proteins; anthocyanins and sugars (36, 57, 61, 111).

There are a few physiological changes which become apparent as plant hardiness increases. Translocation in snap beans is greatly inhibited by a physical blockage of sieve plates in the phloem as the plants harden (21). The efficiency of respiration decreases in hardened plants (42). Increased permeability to water by cellular membranes

has been observed (48, 49, 57). For some as yet unexplained reason, the greater the membrane permeability to water, the greater the resistance to freeze damage.

Questions remain as to the interpretation of the foregoing correlations. Where several factors are mentioned, often one is cited as the primary factor, while the remaining factor's variations are attributed to chance association. The search to uncover a single biochemical alteration to protect against freezing damage may be fruitless. Cellular membranes may be the primary target for this type injury (50). A protein with a molecular weight of about 10,000 has been isolated from chloroplast membranes. It appears to protect the chloroplasts from freezing injury when present in conc as low as 0.05% (27). The available information, however, favors the idea that a number of factors are involved in freeze damage and the protection against this damage (50).

Freezing in Citrus

Plants in the process of cold acclimation typically undergo a biphasic change in general growth and biochemical-physiological patterns. The first step in this process is

the induction of arrested growth or induction of dormancy. Generally dormancy will prevail until a required cold treatment is satisfied. Citrus plants do not require a specific cold treatment in order to resume growth, therefore, in this regard, do not display a true winter dormancy (178). Citrus does, however, exhibit periodic arrested growth. The factors controlling these periods are not precisely known, but they may be related to endogenous growth regulators. Any factor which tends to reduce shoot growth such as photoperiod will influence these dormant periods (8, 11, 12, 44, 117, 173, 179). Concomitant with reduced growth, cool temp cause a reduction in bud and cambial activity (9, 10). Root growth generally ceases when soil temp go below 10°C (10).

The second step in the process of plant acclimation is the development of hardening. The temp regime to which the plants are subjected controls the degree of hardiness (114, 176, 183, 184). As citrus hardiness increases there are increases in total sugars, reducing sugars, % bound water, and electrolyte conc. Simultaneously there are reductions in total water content and water soluble proteins (34, 35, 136, 174, 175, 177, 184).

These are general observations, and the actual process of acclimation needs to be elucidated In citrus acclimation, the final degree of hardiness is quite variable The single most important factor in determining the final hardiness of citrus tissue may be the stability of the bound water in the tissue, which in turn decreases the amount of free water available for nucleation and crystal growth. Through this binding the cellular membranes increase their tolerance to the presence of ice (175, 177, 178).

When citrus trees are subjected to low temp the rate of cooling is dependent on physical and physiological parameters. An average specific heat for fruit has been calculated at approx 0.85 Cal/g/^{o}C, and is variable with the % water content of the fruit. Thermal conductivity for fruit has been calculated at 0.23 BTU·ft/hr·ft^{2}.^{o}F, and is dependent on peel thickness and fruit size (161).

Citrus tissues have the ability to supercool Fruit may supercool to -4.4^{o}C for a short period of time, or to -3.3^{o}C for longer periods. Leaves may supercool to -7.5^{o}C before nucleation takes place (29). Undoubtedly supercooling plays an important role in freeze resistance of citrus (176). Differences in degree of supercooling are noted

among various citrus species. These differences are not significant in developing cold hardiness ratings (172).

Ultimately, during supercooling, nucleation will occur. This final temp is cultivar and tissue dependent. On the average, leaves will freeze at -6.6°C, whereas fruit will freeze at -2.0°C (29). Ice nucleation in leaves has been increased by the accumulation of dew on leaves which, when cooled, turns to frost and acts as nucleation sites (186). Water on the surface of fruit, however, does not appear to increase the incidence of nucleation (70). Ice crystal growth rate is influenced by the growth state of the tissue. Actively growing tissues freeze much more rapidly than hardened citrus tissues (181). Young lemon twigs when frozen display an ice crystal growth rate of about 15cm/min (70).

It has been hypothesized that as the cells are cooled, water moves out of the cell into the intercellular spaces, at which point it freezes, causing the water soaked appearance in leaves (185). Ice formation in citrus tissue may not always be lethal, indicating that killing temp may be 1-2°C lower than the ice nucleation temp (34, 175, 177, 182). Cellular death in citrus was thought to be a result of this water movement out of the cells, causing dehydration

and disruption of membranes involved with the photosynthetic system (176, 177). Reports of damage due to high temp resembling freeze damage, and the recovery of leaves from water soaking have led researchers to believe that dehydration may not be the major factor of damage. Damage to fruit and twigs may be a result of physical disruption of cellular membranes during freezing (59, 162, 185).

Transmission electron micrographs have demonstrated that in freeze damaged citrus tissues there was disruption of the tonoplast and chloroplast membranes, as well as a general disruption of cellular organization. There was damage to the mitochondrial cristae, but the thylakoids remained intact (180, 185). These observations were made by the water-alcohol substitution method. As of yet no one has directly demonstrated intracellular ice formation in citrus (160).

MATERIALS AND METHODS

Plant Materials

Twelve-month-old seedlings of 'Tahiti' lime (C. latifolia Tan.), 'Marsh' grapefruit (C. paradisi Macf.), 'Orlando' tangelo (C. paradisi x C. Reticulata), and 'Owari' satsuma (C. reticulata Blanco) container grown under greenhouse conditions at the University of Florida Gainesville, Florida, were used in these studies. Ten plants of each seedling type were selected for uniformity and healthy appearance. Selected plants were divided into 2 groups; one remaining in the greenhouse to be maintained in an active state of growth, and the other group transported to the U.S. Horticultural Research Laboratory in Orlando, Florida, and placed in a growth chamber programmed to harden citrus plants. The first group of plants were used as a check against the second group

Plants in the growth chamber were conditioned using the environmental parameters developed by Young and Reynado (184). The chamber provides a 12 hr light-dark photoperiod. A diurnal temp scheme is synchronized with the photoperiod.

Temps were maintained at 21.1°C during the light period and 10°C during the dark period for 2 weeks, then lowered to 15.6°C/4.4°C (light/dark) for 2 weeks, and finally 10°C/ -1.1°C for 2 weeks. Temps were accurately maintained to ± 0.56°C. Light intensity in the growth chamber at plant height was 240 microeinstelns (135).

Plants grown in the greenhouse were subjected to 30°C day-time and 18°C night-time average temperatures. Light intensity at noon on a clear day was 800 microeinsteins at plant level (135).

Leaf Freezing Point Determination

The freezing point of leaves from the 4 citrus cultivars was determined by the detached citrus leaf freezing point method described by Gerber and coworkers (20, 33), with modifications incorporated by Hutcheson and Wiltbank (30).

Five leaves were randomly selected from each of the 4 cultivars grown in the greenhouse and the growth chamber Leaves were detached from the plants and freezing point determinations were made immediately The average value of 5 leaves from each cultivar was used as the estimated mean leaf freezing point (MLFP) temp of that cultivar.

Tissue Selection and Transportation

Four leaves per cultivar were randomly selected and detached from the plant. Leaves were stored in specially constructed styrofoam containers 7.5 cm x 12 cm x 10.5 cm with a small rectangle, 1 cm x 6 cm x 6 cm cut out of the middle to accommodate the 4 leaves.

The containers with leaves were then placed in a small portable refrigerator, maintained at 10°C and transported to the laboratory. Transportation time was approx 3 hrs.

Tissue Preparation for Ultra-Thin Sectioning

Six, 1-mm leaf discs were excised from the collected leaves. The discs were fixed in 0.2 M Na cacodylate-6% glutaraldehyde solution (pH 7.2) at 4°C for 1 hr. The fixed samples were washed 3 times in Na cacodylate buffer (pH 7.2), for a total of 30 min, then post fixed in 1% OsO_4 for 1 hr at room temp. The tissue was given a final 3 washings in Na cacodylate buffer at room temp, for a total of 30 min, then serially dehydrated in ethanol and embedded in low-viscosity epoxy according to Spurr's method (144).

Sections were cut on a Sorvall MT-2 ultra microtome with a diamond knife and placed on 0.3% formvar-carbon coated grids, 300 x 75 mesh. Sections were post stained in lead citrate (166) for 10 min.

A Hitachi HU-11-C Transmission Electron Macroscope was used to photograph the sections.

Tissue Preparation for Freeze-Etching

One, 1-mm leaf disc was excised from each of the 4 leaves of each cultivar collected. Discs were then soaked in different solutions to encourage cellular fractures in various dimensional planes. The solutions used were 6% glutaraldehyde for 10-15 min, 20% glycerol for 1 hr, and distilled water for 10-15 min. All solutions were at room temp. Discs were then cut on a diagonal into 8 equal sections and placed in Balzers High Vacuum Corp., No. 11-3654Pi specimen carriers, designed for use in freeze-etch machinery.

Specimen carriers with samples were placed in an insulated styrofoam container which enclosed a Cambion, Model 7S-809-1084-01 thermoelectric cooling plate. This allowed the specimens to be cooled at an average rate of $-4.6^{o}C$/min until the final temp was reached.

Final temp was determined by using the estimated MLFP and selecting a final temp of 2°C above or below that point. The final temp was maintained for 15 min, at which time the specimens were removed from the thermoelectric plate and immediately immersed into liquid Freon 12 (approx -155°C) for 10 sec, then into liquid nitrogen (approx -196°C). The frozen samples were then placed in plastic vials and stored in liquid nitrogen until used.

Freeze-Etching

Previously prepared and stored samples were subjected to freeze-etching on a Balzers Freeze Etching Machine, Model BA-360-M. A modified method of Moor and coworkers was used (100, 101).

Samples were fractured at -110°C and etched for 15-20 sec at the same temp, then immediately replicated.

The replica-specimen combination was removed from the apparatus and soaked overnight in 100% methanol at room temp. Replicas were cleaned of all plant material by placing them in a cleaning solution of 5 g potassium dichromate, 20 ml distilled water, and 100 ml conc sulfuric acid, for about 4 hrs. Cleaned replicas were transferred by wire loop to 2, 8-hr rinses in double-distilled water.

Replices were finelly transferred onto formvar-cerbon coated copper grlds, 300 x 75 mesh. Replicas were photographed using a Hitachi HU-11-C Transmission Electron Microscope.

Treatment Comparisons

Ultra-thin sections of plestic embedded leef tissue from the 4 cultivers were used to compare leef tissue from tender, greenhouse-grown plants to the hardy chamber-grown plents. Observations of celluler morphological changes between the 2 groups of plents were mede.

Samples of double-distilled weter and 6% glutereldehyde were freeze-etched to observe eny ertifects which might be produced. A tissue pretreatment of 20% glycerol was used to elimlnate ice crystel growth within the tissue. This treatment was considered the control.

Tissues subjected to freezing temp of 2°C above end below the estimated MLFP were used to observe the initiel ice crystal formetion and determine the location of early crystal growth

RESULTS AND DISCUSSION

Mean Leaf Freezing Points

MLFP's for greenhouse and growth chamber plants were taken at weekly intervals for a total of 6 weeks. Results are shown in Table 1. Plant cultivars were selected for the experiment on the basis of previous research and observations which indicated that they display varying degrees of cold hardiness (172, 182): 'Tahiti' lime being the most tender, followed by 'Marsh' grapefruit, 'Orlando' tangelo, and finally the hardy 'Owari' satsuma. Experiments were started on 10/7/75 and 12/15/75. Plants in the growth chamber hardened to the same relative degree in each of the 2 experiments. Greenhouse-grown plants demonstrated a MLFP up to 1.5°C lower the second time compared to the first, although the final degree of hardening was not different. Recorded night-time greenhouse temp before the second test were repeatedly in the 9°-12°C range. These temps were apparently sufficient to slightly harden the plants. The final mean leaf freezing points of the chamber grown

Table 1. Mean leaf freezing points (MLFP) of citrus plants acclimated in a growth chamber.

	MLFP[y] (°C)						
	Week						
	0[z]	1	2	3	4	5	6
'Tahiti' lime	-3.9	-4.7	-4.7	-4.5	-4.8	-5.7	-4.3
'Marsh' grapefruit	-5.3	-6.1	-6.3	-7.1	-7.5	-6.2	-7.0
'Orlando' tangelo	-4.8	-5.5	-5.0	-6.2	-5.8	-5.8	-7.7
'Owari' satsuma	-5.7	-6.2	-6.6	-5.8	-6.2	-6.9	-8.4

[y]L.S.D. 5% = 0.9

[z]Greenhouse-grown plants

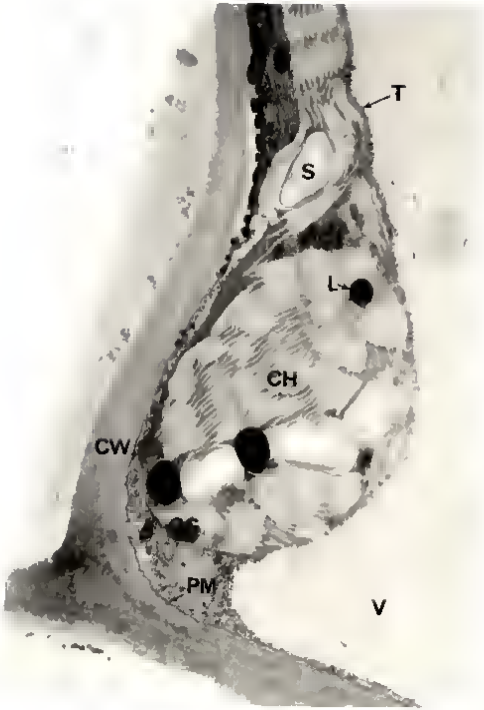
plants were similar to temp previous reported by other workers (30, 183, 184, 185). It was therefore assumed that these plants had been successfully hardened.

Ultra-Thin Sections

Transmission electron micrographs of ultra-thin sections, approx 0.1 μ thick are shown in Figs. 1-8. In comparing hardened vs unhardened leaf tissue, no differences in cellular structure were noted at temp above freezing temp. Cellular organelles are arranged around the periphery of the cell, adjacent to the cell wall, with a large central vacuole. The cytoplasm surrounding the various lipid particles, chloroplasts and mitochondria varied greatly in thickness a situation similar to that reported by other workers (151, 180). In areas adjacent to chloroplasts and nucleus, the cytoplasm becomes relatively thick, while in areas away from cellular organelles, it becomes so thin that the plasma membrane and tonoplast appear to touch.

The chloroplasts of both hardened and unhardened plants displayed membrane systems characteristic of normal chloroplasts, however, the grana stacks were negatively stained. No chloroplast swelling or interthylakoid dilation was observed, as has been reported in some plants

Fig. 1. Electron [illegible] of unhardened [illegible] cells of 'Tahiti' [illegible] (12,400X) CH, chloro[illegible] CW, cell wall; L, lipid particle; OG, osmophilic [illegible]le PM, plasma membrane; S, starch grain, T, [illegible]; V, vacuole


T
S
L
CH
CW
PM
V

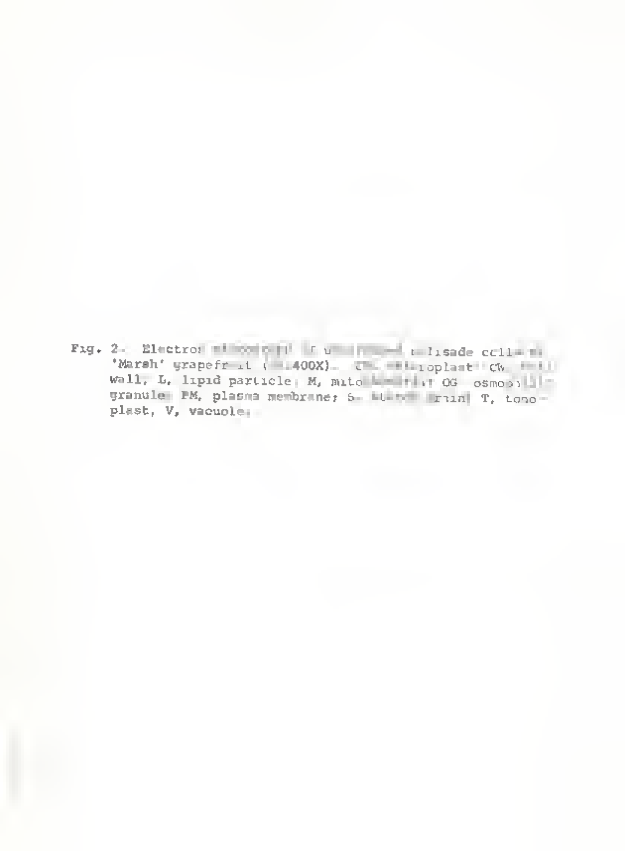


Fig. 2. Electron [illegible] palisade cells [illegible] 'Marsh' grapefruit ([illegible]400X). [illegible] chloroplast; CW, cell wall; L, lipid particle; M, mitochondria; OG, osmophilic granule; PM, plasma membrane; S, starch granule; T, tonoplast; V, vacuole.

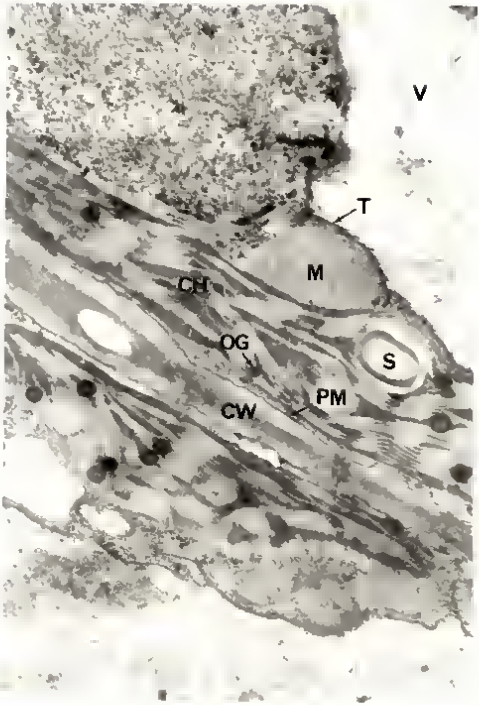

V
T
M
CH
OG
S
PM
CW

Fig. 3. Electron micrograph of unhardened palisade cells of 'Orlando' tangelo (32,400X). CH, chloroplast; CW, cell wall; L, lipid particle; M, mitochondria; PM, plasma membrane; S, starch grain, T, tonoplast, V, vacuole.

M
V
PM
T
CW
S
CH

Fig. 4. Electron micrograph of unhardened palisade cells of 'Owari' satsuma (32,400X). CH, chloroplast; CW, cell wall; L, lipid particle; M, mitochondria; PM, plasma membrane; S, starch grain; T, tonoplast; V, vacuole.

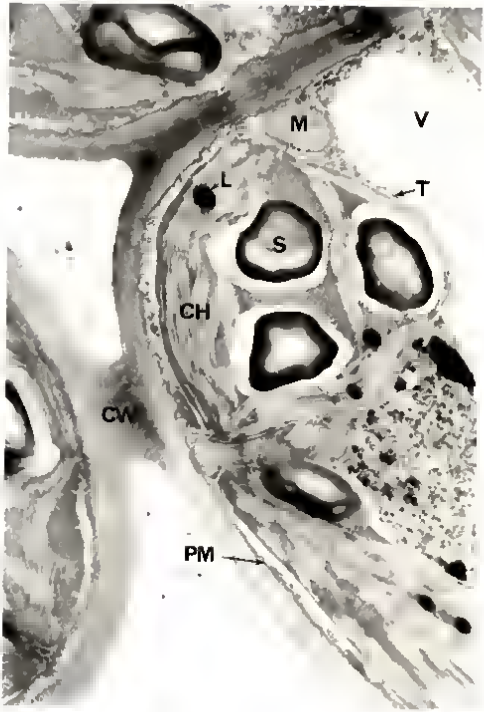

M
V
L
T
S
CH
CW
PM

Fig. 5. Electron micrograph of hardened palisade cells of 'Tahiti' lime (32,400X). CH, chloroplast, CW, cell wall; L, lipid particle, M, mitochondria; PM, plasma membrane; S, starch grain; T, tonoplast; V, vacuole.

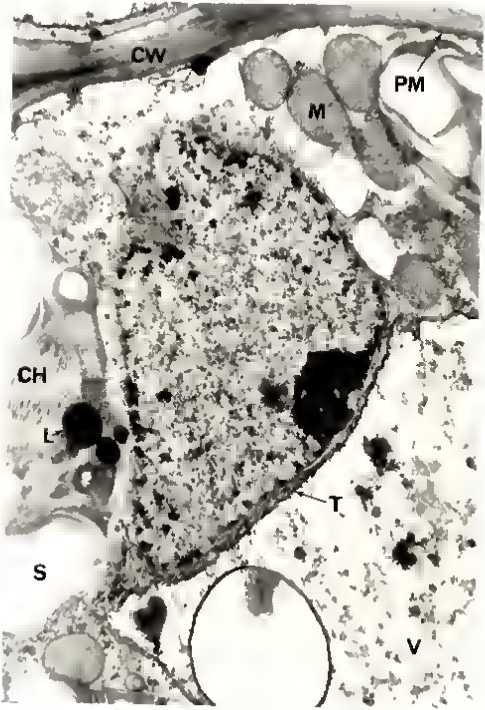

CW
PM
M
CH
L
T
S
V

Fig. 6. Electron micrograph of hardened palisade cells of 'Marsh' grapefruit (32,400X). CH, chloroplast; CW, cell wall; L, lipid particle; M, mitochondria; OG, osmophilic granule; PM, plasma membrane; S, starch grain; T, tonoplast; V, vacuole.

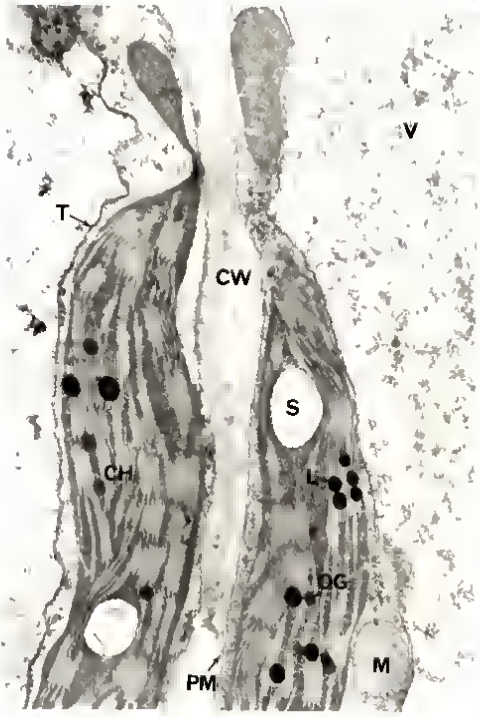

T
CW
V
S
CH
L
DG
PM
M

Fig. 7. [illegible] rograph of hardened palisade cells of [illegible] tuberculo (32,400X). CH, chloroplast; CW, cell wall; L, lipid particle; M, mitochondria; OG, osmophilic granule; PM, plasma membrane; S, starch grain; T, tonoplast; V, vacuole

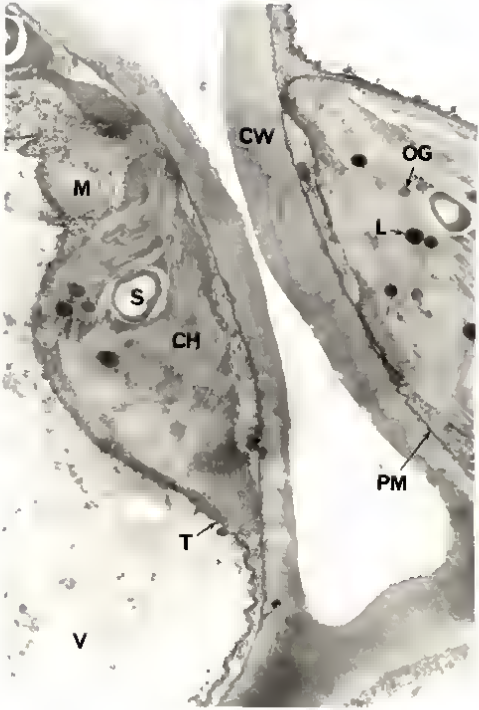
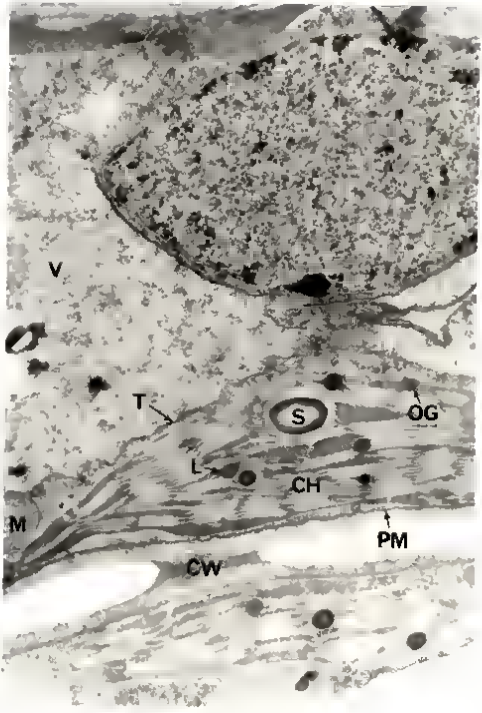

CW
OG
M
L
S
CH
PM
T
V

Fig. 8. Electron micrograph of hardened palisade cells of 'Owari' satsuma (32,400X). CH, chloroplast; CW, cell wall; L, lipid particle; M, mitochondria; OG, osmophilic granule; PM, plasma membrane; S, starch grain; T, tonoplast; V, vacuole.


V
T
S
OG
L
CH
M
PM
CW

(43, 150). Gross observation of starch grain numbers and sizes in the chloroplasts were made. Comparing unhardened to hardened leaf tissues, the starch grains appeared to remain the same relative size. In the same comparison, the total number of starch grains per chloroplast remained approx equal. This could indicate that in citrus, cells subjected to cold hardening environments do not utilize the starch grains as a source of cellular energy, but rather the chloroplasts continue to produce new sugars, which are directly utilized. Previous workers have demonstrated that citrus plants exposed to hardening conditions do exhibit an increase in total and reducing sugar (35, 136, 176, 184). It may be argued that this increase is partially a result of the loss of total cellular water (184), however, it has been clearly demonstrated that photosynthetic activity is present up to the point of cellular freezing (177).

The major cellular membranes, plasma membrane and tonoplast, appeared to be intact in both hardened and unhardened leaf tissue. Both membranes displayed the typical bilayered structure. This structure did not appear altered during the hardening process.

The mitochondria in the unhardened leaf tissues appeared similar to the hardened tissue when comparing density, relative size and general appearance.

Freeze-Etching of Citrus Leaf Tissue

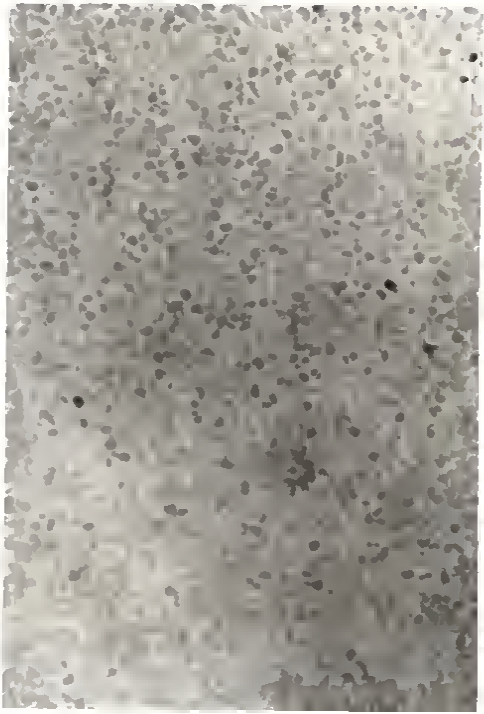
Freeze-Etch Replication of Solutions

A 6% glutaraldehyde solution was subjected to the same freeze-etch techniques as were the leaf samples. The preparation of the solution differed from that of the tissue samples in that the solution was supercooled to ~10.6°C, which represented the lowest temp to which leaf tissue was subjected. The result is shown in Fig. 9. The solution was used as a cellular fixative to help stabilize cellular membranes during destructive ice crystal formation and to act as an indicator of ice formation by modifying ice crystal formation into a lace-like appearance. The frozen glutaraldehyde showed a structure typical of most cryoprotectants (4).

As a comparison double-distilled water was subjected to the same freeze-etch technique and temp. The result is shown in Fig. 10. The resulting large crystal structure was quite unexpected because these shapes did not correspond with previous electron micrographs (14). However, in the

Fig. 9. Freeze-etched replica of 6% glutaraldehyde solution (32,400X).

Fig. 10. Freeze-etched replica of [illegible]-distilled water supercooled to -10.6°C and maintained at that temp for 15 min (32,400X).



experiment of Davy and Brenton (14) the water was rapidly cooled from room temp, whereas in the experiment of this dissertation the water was supercooled to -10.6°C and maintained there for 15 min, during which time no ice formation occurred. When the water-specimen carrier combination was touched with forceps upon removal from the thermoelectric plate to the liquid Freon 12, ice crystal formation took place immediately.

To check this discrepency, double-distilled water was frozen as before, and compared to samples of double-distilled water taken from room temp and placed directly into liquid Freon 12. The supercooled water exhibited the crystal structure shown in Fig. 10, but the room temp to liquid Freon 12 sample exhibited a flat pebbled surface (Fig. 11). The pebbled surface is similar to that reported by Davy and Brenton (14).

The process of supercooling water appears to alter the final crystal shape when subjected to freeze-etch techniques. This observation may be important in that the initial ice crystal size, or the size of the crystals in a growing boundary may depend on the degree of leaf tissue supercooling. Ice formed in leaves which are cooled to a temp where

Fig. 11. Freeze-etched replica of double-distilled water frozen from room temp (32,400X).

ice formation just begins may survive because resulting crystals are extremely small and not mechanically damaging to the cell. In supercooled leaves, resulting ice crystals could be large and mechanically damaging to cellular constituents.

Interestingly, the supercooled water did not nucleate until touched by forceps. This is paralleled by a natural situation where supercooled leaves display no water soaked appearance, when cooled well below the freezing point of water. However, if these leaves are disturbed by touching or shaking, water soaking will be immediately apparent. Water soaking is thought to be a result of intercellular ice formation (185).

Initial Ice Formation in Leaf Tissue at 2°C Above MLFT

Initial ice crystal formation in citrus leaf tissue takes place in the intercellular spaces. In these spaces free extracellular water may be found. In comparing the 4 cultivars tested, freezing first occurred intercellularly in all.

In order to locate initial ice crystal formation a control group was established. This was done by treating the

tissue prior to freezing in 20% glycerol at room temp for 1 hr. This treatment has been demonstrated to almost totally prevent ice crystal formation when cells are cooled rapidly (100). The pretreated tissues were then placed directly into liquid Freon 12, and finally into liquid nitrogen. The results are shown in Figs. 12-15. It can be seen that the cellular constituts are intact with no discernible ice crystal formation or damage, either intra- or intercellularly. Cell walls, plasma membranes and other organelles have remained intact.

A sample group of each cultivar soaked in 6% glutaraldehyde for 15 min at room temp, then frozen at +2°C above the MLFP of greenhouse plants (Table 2) was compared to the control group. The results are shown in Figs. 16-19. These electron micrographs clearly show the presence of intercellular ice and the absence of intracellular ice in all 4 cultivars. The glutaraldehyde served well as an indicator, and appears similar in structure to Fig. 9.

Since this ice appears above the MLFP and below the freezing point of water, its presence clearly supports the theory that initial water spotting is a result of intercellular ice formation, recovery from which is common (185).

Fig. 12. Freeze-etched replica of glycerol pretreated 'Tahiti' lime leaf tissue cell (32,400X) CH, chloroplast, CW, cell wall, V, vacuole.

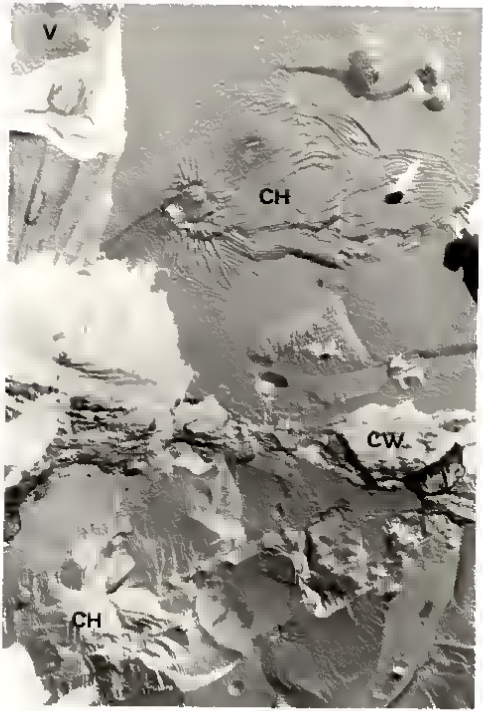

V
CH
CW
CH

Fig. 13 Freeze-etched replica of glycerol pretre
'Marsh' grapefruit leaf tissue cell (32,400X).
chloroplast, CW, cell wall; V, vacuole.

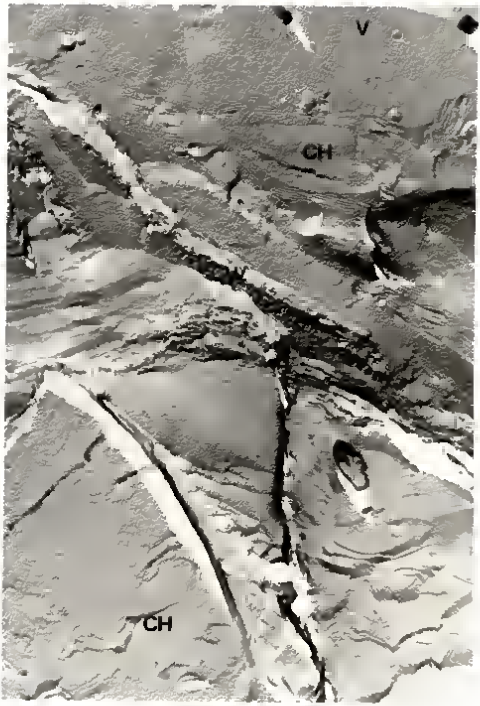

V
CH
CH

Fig 14. Freeze-etched replica of glycerol pretreated 'Orlando' tangelo leaf tissue cell (32,400X). CH, chloroplast, CW, cell wall; L, lipid particles; M, mitochondria; S, starch grain; V, vacuole.

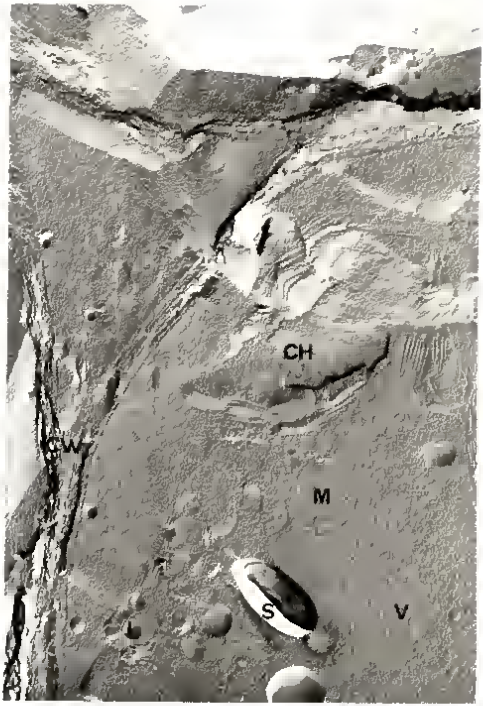

CH
M
S
V

Fig. 15. Freeze-etched replica of glycerol [illegible]
'Owari' satsuma leaf tissue cell (32,400×). [illegible]
chloroplast; CW, cell wall; V, vacuole

V
CH
CW

Table 2. Summary of the final cooling temp maintained on the thermoelectric plate for each citrus cultivar prepared for freeze-etching (°C). Temp shown are differences from the mean leaf freezing point (MLFP) of each variety under 2 sets of environmental conditions.

	Greenhouse Grown		Growth Chamber Grown	
	+2°C	-2°C	+2°C	-2°C
'Tahiti' lime	-3.9	-7.9	-4.5	-8.5
'Marsh' grapefruit	-4.9	-8.4	-5.4	-9.4
'Orlando' tangelo	-5.0	-9.0	-5.6	-9.6
'Owari satsuma	-5.4	-9.4	-6.6	-10.6

Fig. 16. Freeze-fractured section of glutaraldehyde pretreated 'Tahiti' lime leaf tissue frozen at MLPF +2°C (32,400X) CM, chloroplast CW, cell wall I, ice crystals.

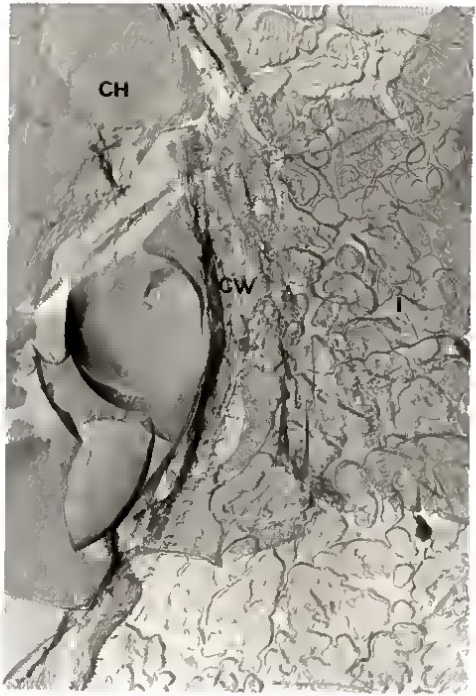

CH
CW
I

Fig. 17. Freeze-etched replica of glutaraldehyde pretreated 'Marsh' grapefruit leaf tissue frozen at MLFP +2°C (32,400X). CW, cell wall, I, ice crystals; V, vacuole.

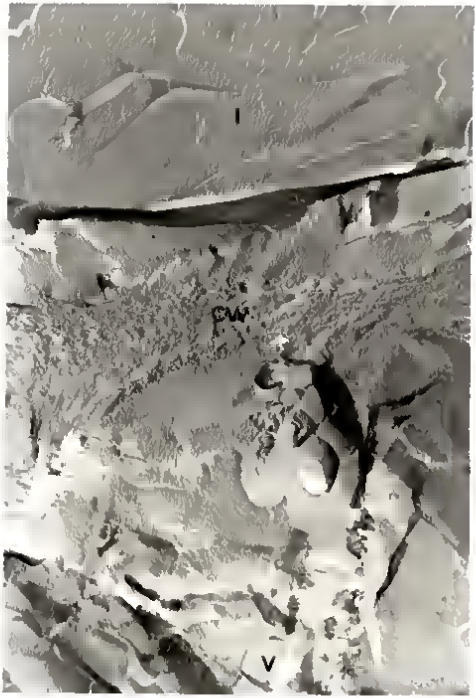

I
V

Fig. 18. Freeze-etched replica of glutaraldehyde pretreated 'Orlando' tangelo leaf tissue frozen at MLFP +2°C (32,400X) CH, chloroplast; CW, cell wall; I, ice crystals.

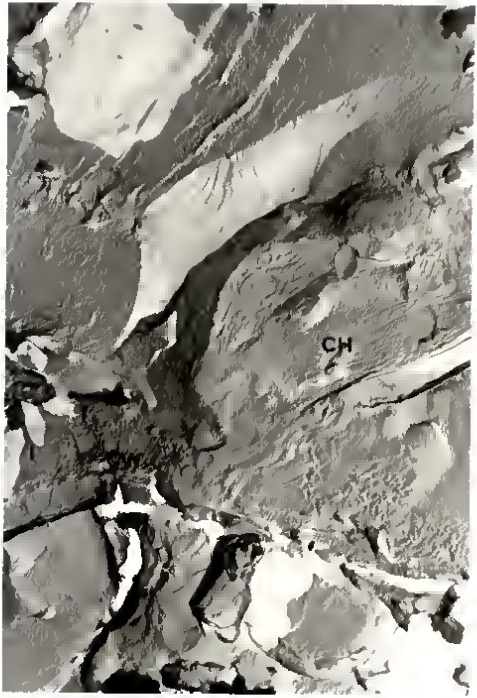
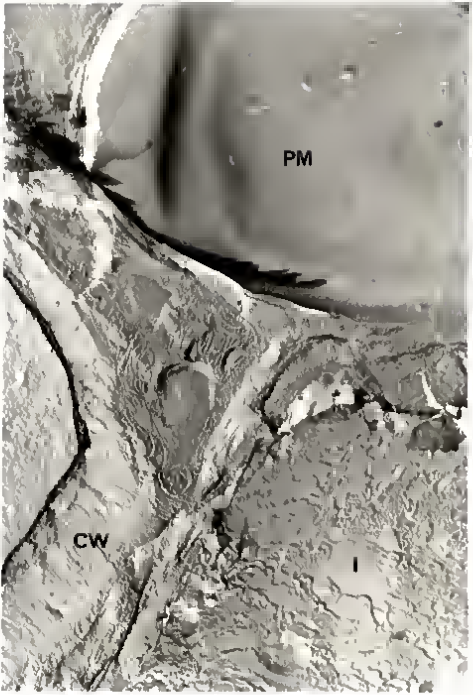

CH

Fig. 19. Freeze-etched replica of glutaraldehyde pretreated 'Owari' satsuma leaf tissue frozen at MLFP +2°C (32,400X). CW, cell wall; PM, plasma membrane; I, ice crystals.


PM
CW
I

The water spotting is a visual effect of ice crystals which act like a series of small fiber optic connections, changing the surface reflectivity of a leaf, thus the dark appearance of water spotting noted in all 4 cultivars.

Intracellular Ice Formation in
Leaf Tissue at 2°C Below MLFP

Figs. 20-23 show the results of subjecting the leaf tissues of the 4 greenhouse-grown cultivars to a temp 2°C below MLFP (Table 2). The results are interesting in that the cultivars of 'Tahiti' lime and 'Marsh' grapefruit demonstrated very little ability to tolerate intracellular ice formation, while the 'Orlando' tangelo and 'Owari' satsuma cultivars resisted intracellular ice formation well at 2°C below their MLFP.

This resistance to intracellular ice follows the general hardiness scheme discussed by other authors in their various works on degree of cold hardiness of citrus (10, 29, 173, 176, 183, 185). These authors list 'Tahiti' lime as very cold tender, with 'Marsh' grapefruit moderately cold tolerant, 'Orlando' tangelo tolerant, and 'Owari' satsuma as very tolerant. The range of tolerance is evident when the plants are hardened in growth chambers and the MLFP

Fig. 20. Freeze-etched replica of glutaraldehyde pretreated 'Tahiti' lime leaf tissue frozen at MLFP $-2^{\circ}C$ (32,400X). CW, cell wall; I, ice crystals.

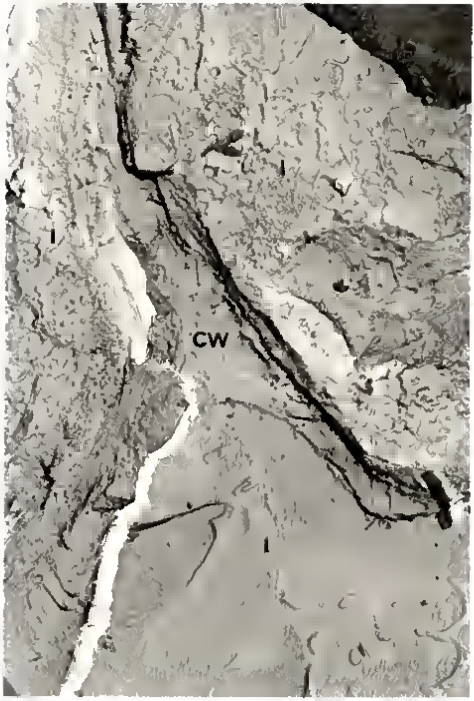

CW

Fig. 21. Freeze-etched replica of glutaraldehyde pretreated 'Marsh' grapefruit leaf tissue frozen at MLFP -2°C (32,400X). CW, cell wall; I, ice crystals.

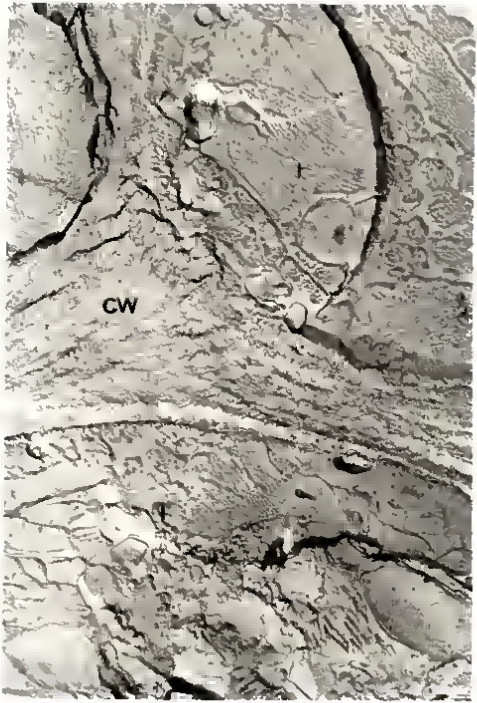
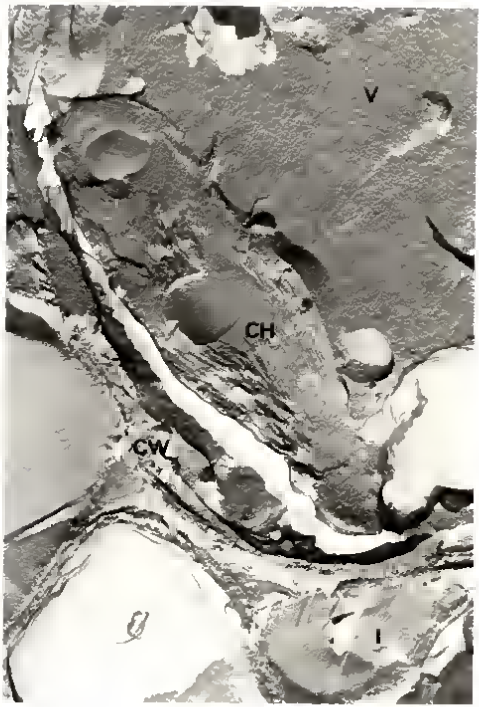

CW

Fig. 22. Freeze-etched replica of glutaraldehyde pretreated 'Orlando' tangelo leaf tissue frozen at MLFP -20C (32,400X). CH, chloroplast, CW, cell wall, I, ice crystals.

CW
CH

Fig. 23. Freeze etched replica of glutaraldehyde pretreated 'Owari' satsuma leaf tissue frozen at MLFP $-2^{\circ}C$ (32,400X). CH, chloroplast; CW, cell wall; I, ice crystals; V, vacuole.


V
CH
CW
I

calculated. Table 1 exemplifies this range: 'Tahiti lime displaying the highest MLFP temp, 'Owari' satsuma the lowest, with 'Marsh' grapefruit and 'Orlando' tangelo in the middle. The actual MLFP numbers indicate that 'Tahiti' lime should probably be grouped by itself, with the other cultivars of colder MLFP grouped together. However, the MLFP is not an indicator of the ability to prevent intracellular ice crystal formation. These numbers were the result of an exothermic temp at which ice formation began, and gave no indication of the ability to survive ice crystal formation or recover from ice damage. 'Tahiti' lime and 'Marsh' grapefruit offer no resistance to intracellular ice formation, whereas 'Orlando' tangelo and 'Owari' satsuma seem to resist intracellular freezing (Figs. 20-23).

When total disruption of the cells occurred, cellular fractures during freeze-etching were poor due to the large ice crystals present. Total disruption of the leaf tissues made good quality replication difficult to obtain.

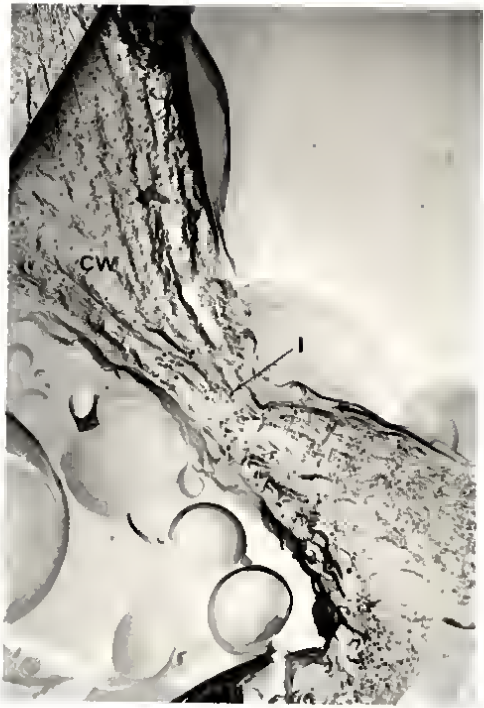
Localized Cellular Disruption
at 2°C Below MLFP

In all cultivars studied some degree of ice crystal formation was noted within the cell wall (Figs. 24-25)

Fig. 24. [illegible]ve-etched [illegible] lime with cell wall intact with [illegible] at 2°C below MLFP (32,400X); CW, cell [illegible] crystals.

CW

Fig. 25. Freeze-etched replica of [illegible] lime exhibiting ice crystals in the cell wall below MLFF (32,400X). CW, cell wall; I, ice crystals.


CW
I

However, its formation appears to be of little consequence with respect to ice crystal damage since no large crystalline growth through the cell wall was observed. The fibrous structure of the cell wall observed with freeze-etching shows small randomly dispersed spaces within the wall. These spaces may be filled with a dilute solution of both electrolytes and nonelectrolytes. Upon freezing ice crystals form in these spaces. Since the spaces are small and segregated, crystal growth is inhibited. The great strength of the cell wall may also physically limit ice crystal growth. The small spaces within the cell wall do not appear to be arranged in a planar dimension through the wall. If water nucleated intercellularly and crystal growth took place, there would be no penetration of the cell wall by a freezing boundary front or single large crystal because there is no channel to follow. These results (Figs. 24-25) indicate that freeze damage at these temps is not the result of mechanical disruption or shearing of cell walls as has been previously theorized (86, 89, 94, 180).

Intracellular ice crystal growth through adjoining membranes and organelles of 'Orlando' tangelo and 'Owari' satsuma was not found. There appeared to be a specific temp

at which ice crystals formed, quite independently in various components of the cell.

Chloroplast tolerance to ice crystal formation was cultivar dependent. 'Tahiti' lime and 'Marsh' grapefruit chloroplasts were totally disrupted at temp 2°C below the MLFF (Figs. 20-21). The disruption was complete, with only the cell wall and starch grains clearly discernible. The chloroplasts of 'Orlando' tangelo and 'Owari' satsuma appeared to resist ice crystal formation under the experimental conditions even though the surrounding cytoplasm contained large ice crystals (Figs. 26-27). No crystalline ice was detected in 'Owari' satsuma chloroplasts frozen to -10.6°C (Fig. 27). The majority of the chloroplasts in 'Orlando' tangelo showed no ice crystal formation, however, a few damaged chloroplasts were observed at -9.6°C (Fig. 28). In undamaged chloroplasts the structure seemed to remain intact, with the thylakoids, grana stacks and chloroplast associated membranes clearly defined. In surface view the outer chloroplast membrane appeared intact. These results appeared at first to contradict previous research by others which relate freeze damage to disruption of the photosynthetic pathway; specifically, CO_2 adsorption, O_2 evolution,

Fig 26. Freeze-etched replica of [illegible] tangelo demonstrating intact chloroplast wit[illegible]rounding frozen cytoplasm at 2°C below MLFP (3[illegible]). CH, chloroplast; CW, cell wall; CY, cytoplasm; I, ice crystals; V, vacuole.

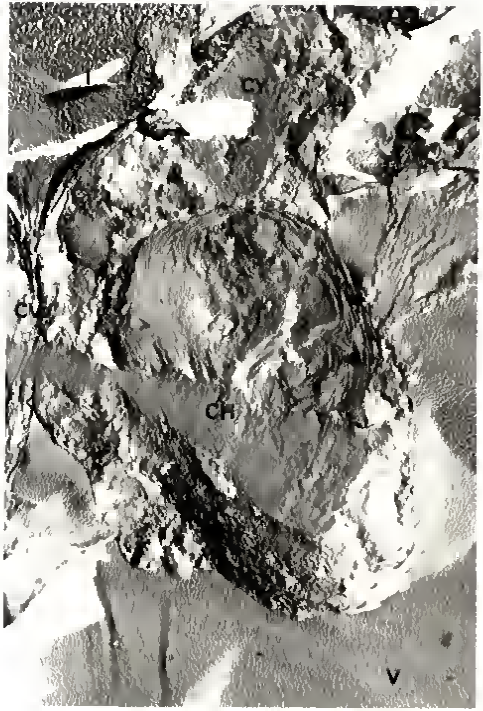

CY
C
CH
V

Fig. 27. Freeze-etched replica of 'Owari' satsuma demonstrating intact chloroplast with surrounding frozen cytoplasm at 2°C below MLFP (32,400X). CH, chloroplast; CW, cell wall; CY, cytoplasm; I, ice crystals; V, vacuole

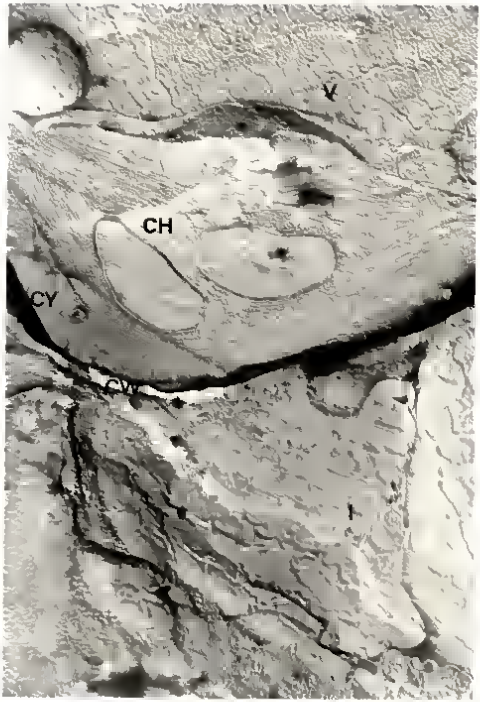
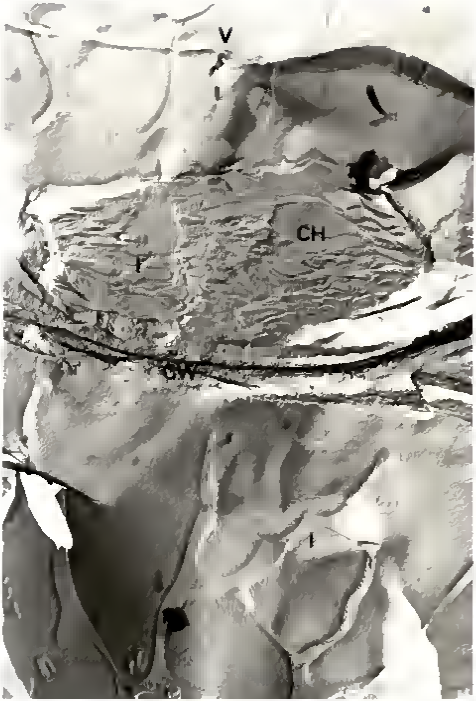

V
CH
CY
CY

Fig. 28. Freeze-etched replica of Citrus tangelo demonstrating ice crystal damage in a chloroplast frozen at 2°C below MLFP (32,400X). Chloroplast CW, cell wall; I, ice crystalline V, vacuole.


V
CH
T
CW
I

ATP synthesis, and electron transport uncoupling (26, 27, 28, 180, 184). The observations in this research were performed on material prepared at temp very close to the isothermic freezing point of the tissue and relate only to the presence or absence of ice crystals in chloroplasts. No attempt was made to determine if the chloroplasts were still functional. Young and Mann's observations (180) describing disrupted chloroplasts of sour orange leaf tissue when frozen may be reconciled by the fact that the tissues were frozen for a longer period of time at a greater temp below the MLFP of the tissue. The greater time and temp may ultimately form crystalline ice within the chloroplasts. The results of actual measurements of photosynthetic substrate changes in relation to freezing (26, 27, 28, 184) may be explained by the fact that some of these experiments were conducted at temp known to be lethal to the tissue. The investigators were not concerned with survivability but with what photosynthetic changes occur at lethal temp. A comparison of this work and that of Young and Paynado (184), performed at relatively the same temp, raises the possibility that even though there is no ice crystal damage in the chloroplasts, they may not be functional. Some temp labile function may

le the disruptive agent in chloroplast damage instead of ice crystal formation. This would account for the fact that totally water soaked leaves have the ability to recover.

A hypothesis of what may happen has intercellular water freezing first, forming a heat sink and establishing a temp gradient from the interior to the exterior of the cell. As various cellular constituents cool they ultimately reach a specific temp at which nucleation begins. As ice crystals form within a specific area of the cell there is a release of heat absorbed by the surrounding cellular structures, which in turn releases the heat through the heat gradient back to the intercellular ice. The overall result is that with the 'Tahiti' lime and 'Marsh' grapefruit cultivars, once nucleation begins within the cell, it proceeds rapidly throughout the entire cell (Figs 20-21). This is due to either a very high heat transfer coefficient which would move great amounts of heat rapidly across the heat gradient, or that cellular constituents of the 2 cultivars freeze at relatively the same temp. 'Orlando' tangelo and 'Owari' satsuma display distinct areas of freezing, with organelles such as the chloroplasts escaping ice crystal formation at the temp investigated. This is probably a

result of the variable freezing points of the cellular constituents of these cultivars.

Membrane Disruption of Hardy Varieties

Plasmalemma disruption in 'Orlando' tangelo and 'Owari' satsuma cultivars was observed (Figs. 29-30). In comparing the control (Figs. 31-32) to the disrupted plasmalemma, it is noted that the disrupted areas appear to be clustered rather than a general disruption of the membrane. This disruption undoubtedly accounts for the previously observed leakage of electrolytes from the cell upon freezing (177).

Greenhouse-grown leaf tissue from each cultivar was soaked in double-distilled water for 15 min at room temp, then frozen at 2°C below the MLFP. This water pretreatment encouraged deep etching and membrane fractures.

At this temp the cells of the lime and grapefruit varieties were totally disrupted with large ice crystals and replicas contained no useful information. Replicas of the hardy tangelo and satsuma varieties demonstrated fractures along the central hydrophobic plane of the membrane bilayer. Similar observations have been made by other workers (22, 115, 146). The plasmalemma of 'Orlando' tangelo exhibited clustered integral proteins rather than the

Fig 29 Freeze-etched replica of 'Orlando' tangelo frozen at MLFF -2°C in water exhibiting plasma membrane damage (32,400X). CW, cell wall D, damaged area; I, ice crystals, PM, plasma membrane

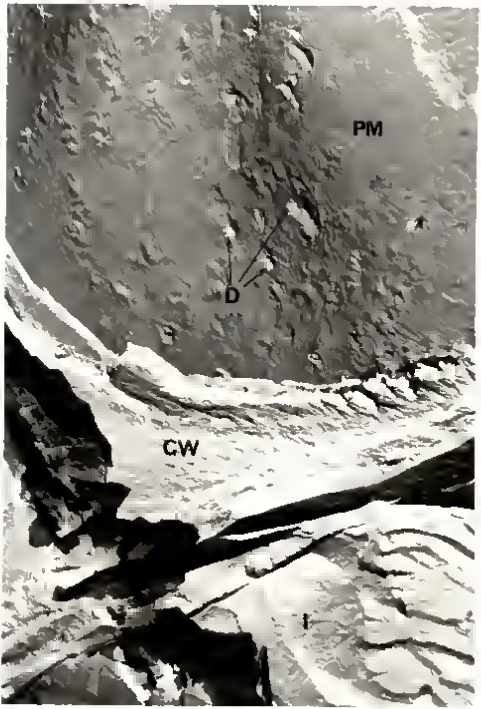

PM
D
CW
I

Fig. 30. Freeze-etched replica of 'Owari' satsuma frozen at MLFP -2°C in water exhibiting plasma membrane damage (32,400X). CW, cell wall; D, damaged area; I, ice crystals; PM, plasma membrane.

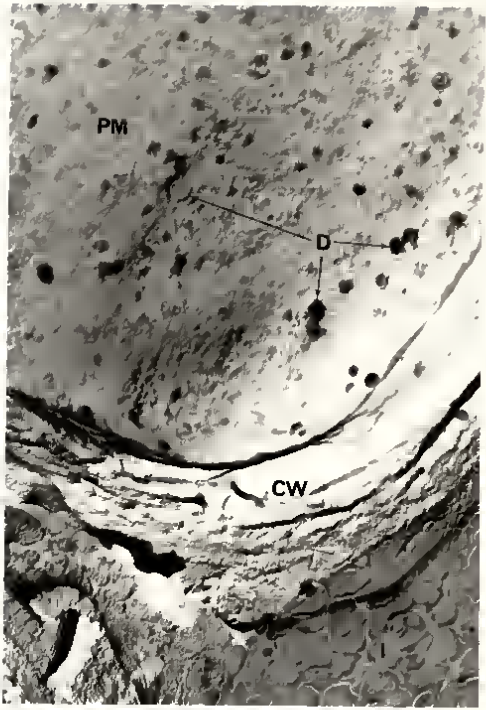
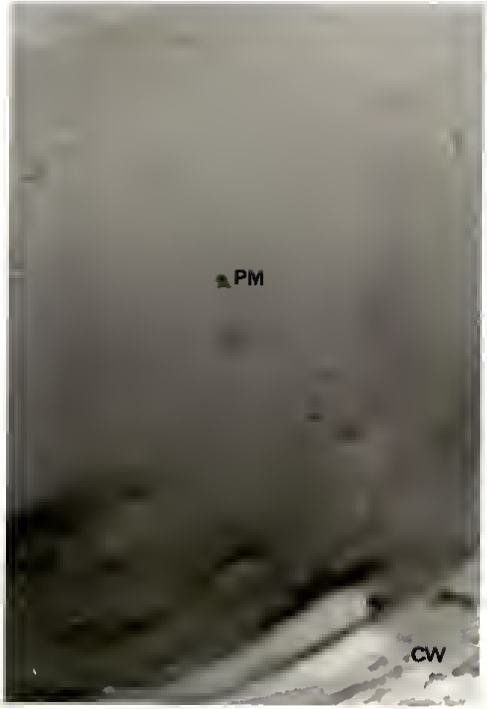

PM
D
CW

Fig. 31 Freeze-etched replica of 'Orlando' tangelo glycerol control showing intact plasma membrane (32,400X). CH, chloroplast; CW, cell wall; PM, plasma membrane.

CH
PM
CW
PM

Fig. 32. Transmission electron micrograph of 'Owari' satsuma glycerol control showing intact plasma membrane (54,000X). CW, cell wall; PM, plasma membrane.


PM
CW

normal evenly distributed particles of the other cultivars. This was probably strictly a phenomena of cultivar difference.

SUMMARY AND CONCLUSIONS

Seedling plants of 'Tahiti' lime, 'Marsh' grapefruit, 'Orlando' tangelo and 'Owari' satsuma were divided into 2 groups, 1 grown in a greenhouse, the other cold hardened in a growth chamber. MLFF were taken at weekly intervals for a total of 6 weeks to monitor the process of hardening. The 2 groups were used to compare the hardened to unhardened plant material, and observe any differences due to hardening.

Ultra-thin sections of hardened and unhardened plants of each cultivar were prepared and viewed by a transmission electron microscope. No differences in cellular constituents were observed between the 2 groups.

Tissues of the hardened and unhardened groups were precooled to 2°C above and 2°C below the MLFF for each cultivar by use of a thermoelectric cooler, then rapidly cooled and stored in liquid nitrogen. Freeze-etched material that had been treated at 2°C above the MLFF displayed initial ice formation only in the intercellular spaces of the leaf tissue.

Intercellular ice formation was observed in all cultivars tested, and it is apparently the cause of the water soaked appearance in citrus leaves at subfreezing temp.

The degree of damage to leaf tissues of the 4 cultivars subjected to temp 2°C below the MLFP of each variety coincided with generally accepted degree of cold tolerance between the cultivars. 'Tahiti' lime and 'Marsh' grapefruit tissues were totally disrupted by large ice crystal formation which made replication extremely difficult. 'Orlando' tangelo and 'Owari' satsuma displayed only minor intracellular disruption.

In all cultivars some degree of ice crystal formation was observed withis the cell walls. These crystals proved to be of little consequence with regard to cellular disruption since no large crystalline ice was observed penetrating through the cell wall into the intracellular area. Rather, the crystals were very small and randomly scattered throughout the cell wall structure.

Large ice crystal growth through adjoining tissues, membranes and organelles was not found. There appeared to be a specific temp at which nucleation and subsequent small ice crystal formation began and this temp was different in

the various cellular constituents. Once the temp of a particular area reached the freezing point of that cell constituent, nucleation occurred.

Chloroplasts of 'Orlando' tangelo and 'Owari' satsuma displayed a very high degree of tolerance to ice formation. The majority of chloroplasts demonstrated no ice crystal damage even though the surrounding cytoplasm clearly was frozen. Chloroplasts of 'Owari' satsuma precooled to -10.6°C exhibited no visible signs of damage to the thylakoids, grana stacks, intergrana lamella and other membranes.

Plasmalemma disruption was observed in all cultivars at 2°C below the MLFP of each cultivar. The disruption in 'Tahiti' lime and 'Marsh' grapefruit cultivars was massive. The hardy 'Orlando' tangelo and 'Owari' satsuma cultivars exhibited scattered damaged areas in the plasmalemma, unlike the totally disrupted cultivars.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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